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A Functional Genomics Approach to Tanshinone Biosynthesis Provides Stereochemical Insights

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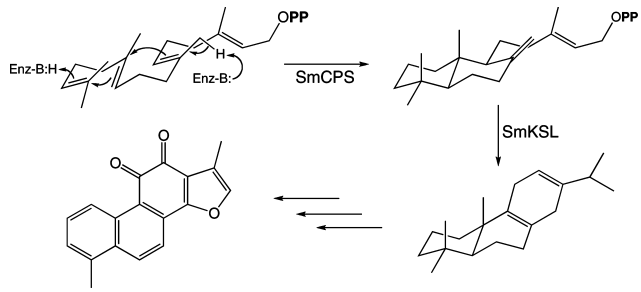
ABSTRACT



Tanshinones are abietane-type norditerpenoid quinone natural products that are the bioactive components of the Chinese medicinal herb *Salvia miltiorrhiza* Bunge. The initial results from a functional genomics-based investigation of tanshinone biosynthesis, specifically the functional identification of the relevant diterpene synthases from *S. miltiorrhiza*, are reported. The cyclohexa-1,4-diene arrangement of the distal ring poises the resulting miltiradiene for the ensuing aromatization and hydroxylation to ferruginol suggested for tanshinone biosynthesis.

Tanshinones are abietane-type norditerpenoid quinone natural products found in the Chinese medicinal herb *Salvia miltiorrhiza* Bunge. Specifically, they are found as bioactive lipophilic pigments in the intensely red rhizome (root), which is called danshen in Chinese traditional medicine, with records of its use going back millennia. More recently, the predominant and more intensely studied tanshinones I (**1**), IIA (**2**), and IIB (**3**) and cryptotanshinone (**4**) have been isolated and found to have a variety of pharmaceutical activities, including antibacterial, antiinflammatory, and anticancer properties.[1] However, while considered abietane-type diterpenoids, the stereochemistry of the relevant abietadiene olefin intermediate and, hence, other derived biosynthetic intermediates is obscured by the aromatic nature of the identified tanshinones (Figure 1). Resolution of the configuration of this intermediate is a critical step in the characterization of tanshinone biosynthesis.

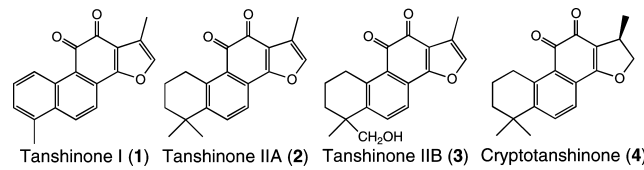


Figure 1. Major tanshinones (**1**−**4**) found in *S. miltiorrhiza*.

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Tanshinones fall into the labdane-related class of diterpenoids, whose biosynthesis is uniquely initiated by a sequential pair of cyclization reactions. The characteristic fused bicyclic hydrocarbon structure is formed from the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP, **5**) in an initial carbon−carbon double-bond protonation-initiated reaction catalyzed by class II diterpene cyclases. These typically form labdadienyl/copalyl diphosphate (CPP), with the corresponding enzymes then termed CPP synthases (CPS). It is at this step that the initial stereochemistry (i.e., of CPP) is established, which is designated by comparison to that of the analogous A/B ring substructure in sterol biosynthesis (i.e., by normal, *ent*, *syn*, or *ent-syn*).[2] Additional stereocenters also are generally formed in the subsequent cyclization and/or rearrangement reaction catalyzed by CPP-specific class I diterpene synthases,[2] which are often termed kaurene synthase-like (KSL) because of their similarity to the kaurene synthase found in all higher plants for the requisite biosynthesis of gibberellin phytohormones.[3]

To enable a functional genomics-based approach to tanshinone biosynthesis, a cDNA library was constructed from *S. miltiorrhiza* root tissue. To take advantage of the inducible nature of tanshinone biosynthesis,[4] a microarray chip was manufactured from ~8700 random cDNA inserts, which ranged in size from 0.5 to 2.5 kb. This was used to compare mRNA levels from induced and control *S. miltiorrhiza* hairy root cultures. Of the clones upregulated by elictor treatment, only one CPS homologue and one KSL homologue were found, both as partial cDNA clones. These also were determined to be the only such homologues in the microarray. Given the importance of the corresponding enzymatic reactions in initiating tanshinone biosynthesis and fixing the stereochemical configuration of the subsequent metabolism, these putative *S. miltiorrhiza* diterpene synthases were chosen for analysis. Thus, the missing sequence for each (SmCPS and SmKSL) was obtained via rapid amplification of the cDNA ends, and the corresponding full-length mRNA sequence was determined.[5]

Plant secondary metabolism, such as tanshinone biosynthesis, is generally regulated by transcriptional control of the genes encoding the relevant enzymes. Thus, both SmCPS and SmKSL seemed likely to be involved in tanshinone biosynthesis because their mRNA levels are increased >2-fold by elicitation, application of a biotic−abiotic combination of the carbohydrate fraction of yeast extract with Ag^+, which has previously been shown to induce tanshinone production.[4] This was further examined through application of the plant defense signaling molecule methyl jasmonate (MeJA), which also was found to increase both the mRNA levels of these diterpene synthases and, subsequently, tanshinone IIA (**2**) biosynthesis in *S. miltiorrhiza* hairy root cultures (Figure 2). The observed coinduction by two separate

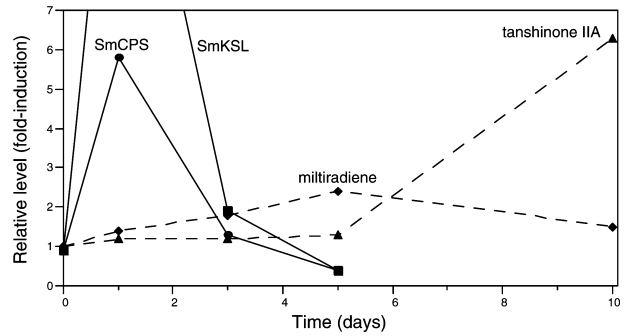

Figure 2. Relative (fold-induction) levels of SmCPS (squares) and SmKSL (circles, 15-fold at 1 day) mRNA and the amounts of the derived miltiradiene (**9**, diamonds) and presumably downstream tanshinone IIA (**2**, triangles) found in MeJA treated versus control hairy root cultures of *S. miltiorrhiza*.

treatments and increase in mRNA prior to tanshinone accumulation, similar to the analogous temporal pattern observed with rice labdane-related diterpenoid phytoalexin biosynthesis,[3,6] indicate that SmCPS and SmKSL may be involved in tanshinone biosynthesis. On this basis, the encoded enzymes were further characterized.

The SmCPS full-length open reading frame was subcloned into pET32a(+) for recombinant expression in *Escherichia coli* (see the Supporting Information for the Materials and Methods section). This SmCPS construct was expressed and purified, via use of the encoded hexahistidine tag, and then assayed for class II diterpene cyclase activity with **5**. The resulting product was enzymatically dephosphorylated for gas chromatography−mass spectrometry (GC−MS) analysis. A comparison to similarly dephosphorylated *ent*- and *syn*-CPP demonstrated that SmCPS-produced CPP of either *ent* or normal stereochemistry (Figure S1 in the Supporting Information). The absolute configuration of the SmCPS-produced CPP was resolved by use of a previously described modular metabolic engineering system,[7] much like that previously described for other class II diterpene synthases.[8] In particular, a pseudomature version of SmCPS (i.e., without the plastid targeting prepeptide) was coexpressed with a GGPP synthase and diterpene synthases specific for normal and *ent*- or *syn*-CPP, with the expected diterpene only obtained with the normal CPP specific enzyme. Thus, SmCPS produces CPP of normal stereochemistry (**6**; Scheme 1). Notably, while normal (5*S*,9*S*,10*S*)-CPP is transiently formed by the bifunctional diterpene synthases involved in gymnosperm resin acid biosynthesis,[9] SmCPS appears to be

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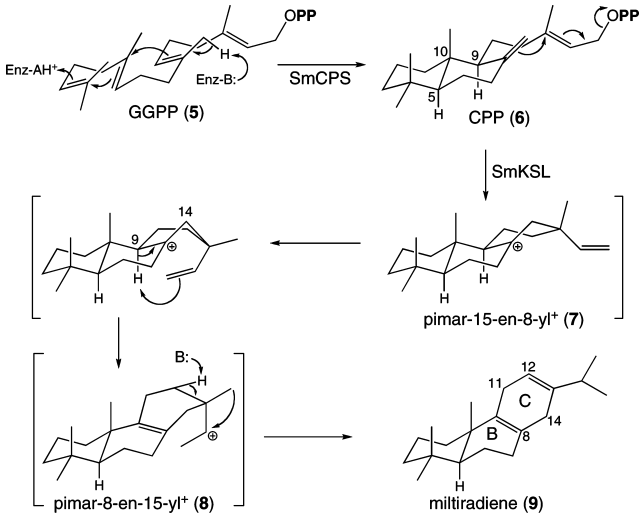
Scheme 1. SmCPS- and SmKSL-Catalyzed Reactions[a]



[a] Cyclization of GGPP (**5**) to normal CPP (**6**) catalyzed by SmCPS and subsequent further cyclization and rearrangement of **6** to miltiradiene (**9**), presumably via pimar-15-en-8-yl$^+$ (**7**) and pimar-8-en-15-yl$^+$ (**8**) intermediates, catalyzed by SmKSL.

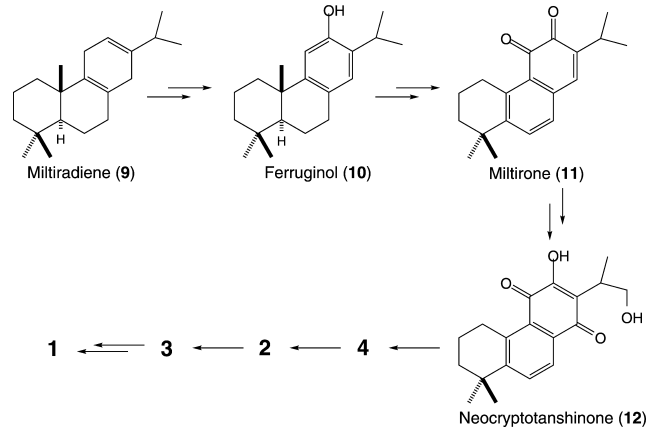
the first identified normal CPP specific CPS and the first class II diterpene cyclase with this particular stereospecificity from an angiosperm.

The catalytic activity of SmKSL was similarly investigated, i.e. first in vitro using recombinant protein expressed from a pET32a(+)-derived construct in *E. coli* and purified via the encoded hexahistidine tag. In coupled assays, this recombinant SmKSL was found to accept the (normal) CPP (**6**) product of SmCPS to produce an unknown diterpene (Figure S2 in the Supporting Information). The identity of this compound was established by use of the same modular metabolic engineering system,[7] much like that described for other novel class I diterpene synthases.[10] Specifically, by production of ~5 mg of the SmKSL product derived from **6**, enabling straightforward analysis by NMR (Figures S3−S8 and Table S1 in the Supporting Information). The structure was assigned using HMBC, HSQC, and COSY data, which demonstrated that this was an abietane-type diterpene, which we propose to name miltiradiene (**9**). Of particular note is the cyclohexa-1,4-diene structure of the distal C ring in **9**, whose double-bond arrangement was evident in the relatively high chemical shifts observed for the protons on the doubly allylic C11 and C14 (i.e., due to deshielding effects), with the presence of only a single methine (i.e., C12) being confirmed by examination of the HSQC data.

While mixtures of abietadienes are produced by some bifunctional diterpene synthases involved in gymnosperm resin acid biosynthesis,[11,12] SmKSL appears to be the first identified normal CPP specific KSL and the first abietane-type diterpene producing class I diterpene synthase of any kind identified from an angiosperm. In addition, the observed 8,12-diene arrangement in **9** presumably requires a distinctly different configuration of the intervening pimar-15-en-8-yl$^+$ (**7**) intermediate in SmKSL, specifically, to enable proton transfer from C9 (Scheme 1) and formation of pimar-8-en-15-yl$^+$ (**8**) rather than proton transfer from C14 to form pimar-8(14)-en-15-yl$^+$, as was previously demonstrated for the bifunctional gymnosperm abietane-type diterpene synthases.[13,14]

As expected from the inducible nature of the relevant SmCPS and SmKSL, an increase in **9** is observed in *S. miltiorrhiza* hairy root cultures following MeJA treatment (Figure 2). Notably, **9** only transiently accumulates, and its subsequent decrease is inversely related to an increase of tanshinone IIA (**2**), suggesting that **9** is an intermediate en route to the tanshinones (**1**−**4**). Given the normal stereochemistry defined by **9**, it seems likely that the previously observed production of ferruginol (**10**) by *S. miltiorrhiza* is also relevant to tanshinone metabolism, and consideration of other identified diterpenoid natural products,[1] specifically miltirone (**11**) and neocryptotanshinone (**12**), enables the proposal of a hypothetical biosynthetic pathway, albeit still incomplete (Scheme 2).

Scheme 2. Hypothetical Tanshinone Biosynthetic Pathway



Interestingly, while the bifunctional abietane-type synthases from gymnosperms produce mixtures of conjugated double-bond abietadienes, SmKSL is quite specific in its production of **9**, which is found as >95% of the total product output. The apposing 1,4-diene arrangement in **9** imposes planarity on the C ring and across the B/C ring bridgehead, just as is found in the aromatic tanshinones and the other identified diterpenoid natural products from *S. miltiorrhiza*.[1] Accordingly, such planarity appears to be imposed early in tanshinone biosynthesis and utilized by the subsequently acting biosynthetic enzymes. Indeed, cyclohexan-1,4-dienes such as that observed in the C ring of **9** are generally

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considered relatively unstable and will readily aromatize, indicating that **9** is poised for further relevant transformations, specifically aromatization and hydroxylation to form ferruginol (**10**).

Regardless of the exact series of relevant transformations, stereochemical resolution of **9** as a presumably relevant intermediate enables investigation of the critical downstream tanshinone biosynthetic enzymes, which will be further enabled by the molecular tools reported here. Thus, the functional characterization of SmCPS and SmKSL reported here has identified a novel normal stereochemistry specific CPS and subsequently acting miltiradiene synthase (SmKSL) and laid the basis for further investigation of tanshinone biosynthesis.

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Supporting Information Available: NMR and MS data for **9** and the Material and Methods section. This material is available free of charge via the Internet at http://pubs.acs.org.

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